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# Fluoroaluminate mimics agonist application in single rat hepatocytes

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Single rat hepatocytes microinjected with the photoprotein aequorin were stimulated with glycogenolytic agonists or low concentrations of fluoroaluminate. Both protocols resulted in the generation of oscillations in cytosolic free $Ca^{2+}$ levels from a resting value of approx. 200 nM and peaking at over 600 nM. However, oscillations induced by receptor-dependent agonists were more regular in both frequency and time course than those induced by direct activation of G-proteins. The role of G-proteins in the generation of repetitive free $Ca^{2+}$ oscillations is discussed.

## INTRODUCTION

Heterotrimeric guanine nucleotide binding proteins (G-proteins) provide the transducing link between agonist–receptor interaction at the cell surface and stimulation of phosphoinositidase C (PIC) activity [1,2]. Activation of this pathway leads to the generation of inositol trisphosphate and 1,2-diacylglycerol from phosphatidylinositol 4,5-bisphosphate. Changes in cytosolic free calcium levels (free Ca), dependent on inositol trisphosphate production, have been found to be oscillatory in a number of cell types [3], including hepatocytes [4]. Hepatocyte free Ca transients exhibit an agonist-specified time course, and the frequency of a series of transients depends on agonist concentration [5].

The sequence of steps involved in G-protein activation is well understood; see [6–8] for reviews. Differential sensitivity to ADP-ribosylation by bacterial toxins [7,9, 10] and stimulation of activity by non-hydrolysable GTP analogues such as guanosine 5′-[γ-thio]triphosphate (GTPγS) and fluoroaluminate ($AlF_4^-$) have proved to be valuable tools in studying G-protein heterogeneity and the role of G-proteins in signal transduction. Studies on populations of hepatocytes [11] using $AlF_4^-$ have demonstrated the ability of this agonist to induce inositol trisphosphate formation, mobilize intracellular calcium and activate phosphorylase. Here we report the results of studies using $AlF_4^-$ to investigate the mechanism of repetitive free Ca transient generation in single aequorin-injected rat hepatocytes.

## MATERIALS AND METHODS

The methods employed in this study for isolating single rat hepatocytes, the microinjection procedure and the data sampling and analysis procedures have been described previously [4]. The experimental medium consisted of Williams Medium E to which agonists and $AlF_4^-$ were added, either separately or together, at the concentrations stated. Final $AlF_4^-$ concentrations were obtained by diluting stocks of 10 mM-$AlCl_3$ into Williams Medium E to which had previously been added sufficient solid NaF to give the final desired $F^-$ concentration, thus avoiding $CaF_2$ precipitation associated with concentrated stock solutions [1].

Statistical examination of the data was undertaken using the Students *t*-test; significance was assumed when $P < 0.05$. All results are expressed as means ± S.E.M.

## RESULTS

Figs. 1(*a*) and 1(*b*) show a comparison of the effects of superfusing a single aequorin-microinjected rat hepatocyte with phenylephrine (2 μM) and then $AlF_4^-$ (1 μM-$Al^{3+}$/1 mM-$F^-$). The oscillations induced by $AlF_4^-$ were more irregular than those induced by physiological glycogenolytic agonists (see [4,5,12]). The transient shapes varied from simple phenylephrine-like oscillations to more complex forms with multiple oscillations on the falling phase, reminiscent of those induced by peptide hormones or $P_{2y}$-purinoceptor agonists [12,13]. The $AlF_4^-$-induced transient shapes were not influenced by prestimulating the cells with phenylephrine. Stimulating cells with low $AlF_4^-$ concentrations and then phenylephrine resulted in normal free Ca transients (results not shown). The duration of $AlF_4^-$-induced transients varied from approx. 5 s to over 150 s; in addition, periodicity was also irregular, single transients or groups of multiple oscillations being quite common (Fig. 1*b*). The peak free Ca level attained during any series of transients also fluctuated, ranging from approx. 500 nM to over 1100 nM, whereas receptor-dependent transients reached a peak at approx. 700 nM free Ca. The rate of rise, however, during $AlF_4^-$ stimulation was significantly slower than normal, being $352 \pm 46$ nM·$s^{-1}$ (mean ± S.E.M.) for $AlF_4^-$ ($n = 51$) and $547 \pm 46$ nM·$s^{-1}$ (mean ± S.E.M.) for phenylephrine ($n = 49$) ($P < 0.005$). The frequency of the transients induced by receptor-independent stimulation showed concentration-dependence; increasing the $AlF_4^-$ concentration induced more frequent free Ca oscillations (results not shown). Fig. 2 shows details of the time courses of $AlF_4^-$- (5 μM-$Al^{3+}$/5 mM-$F^-$) induced transients, illustrating the variability in shape and frequency.

Abbreviations used: $AlF_4^-$, fluoroaluminate; G-proteins, guanine nucleotide binding proteins; PIC, phosphoinositidase C.

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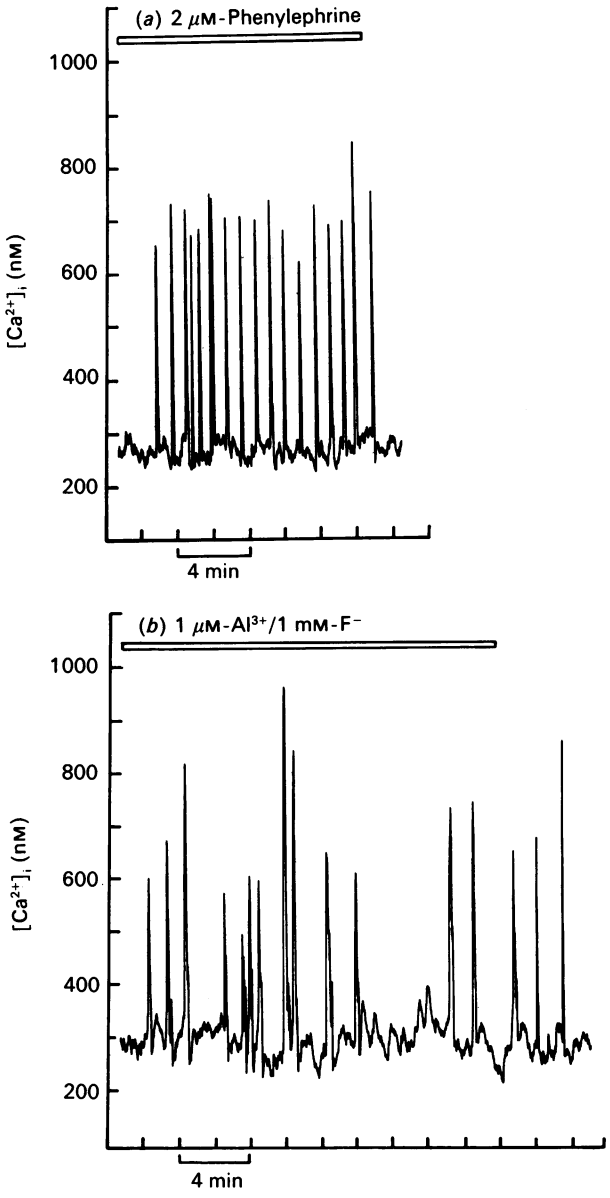


**Fig. 1. Effect of phenylephrine and $AlF_4^-$ on free Ca levels in a rat hepatocyte**

A single rat hepatocyte was microinjected with aequorin and superfused with either agonist or fluoroaluminate at the times indicated. (*a*) The effect of 2 μM-phenylephrine on free Ca levels; (*b*) $AlF_4^-$ (1 μM-$Al^{3+}$/1 mM-$F^-$)-induced free Ca transients. The results shown are typical of five experiments. Time constants: for transients, 1 s; for resting, 20 s.

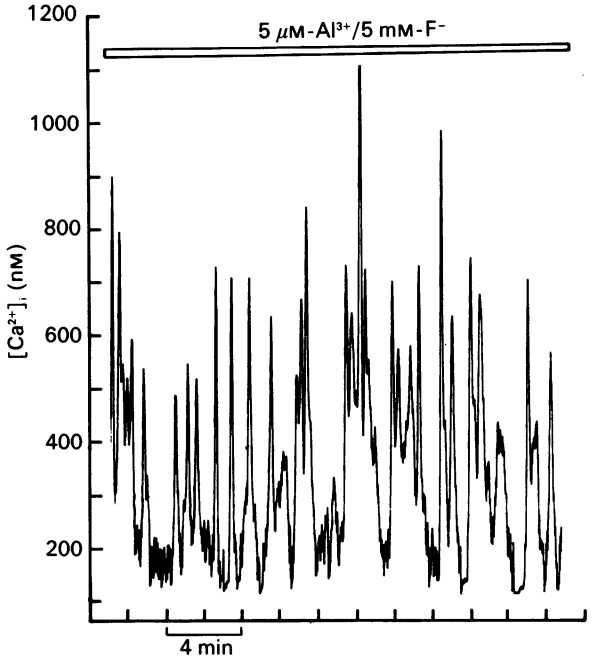


**Fig. 2. Free Ca transients induced by $AlF_4^-$ (5 μM-$Al^{3+}$/5 mM-$F^-$) in a single rat hepatocyte**

Time constants: for transients, 1 s; for resting levels, 15 s.

The delay between starting to superfuse $AlF_4^-$ and the first free Ca transient was comparable with the lag seen with hormones, of the order of 40 s (precise measurement is not possible owing to dead-space in the perfusion chamber). Removal of $AlF_4^-$ resulted in prompt cessation of free Ca transients (within approx. 3–4 min), a longer time than that seen during hormonal wash-off. Cessation of $AlF_4^-$-induced transients was similarly prompt whatever the duration of exposure to $AlF_4^-$.

In four cells, adding low $AlF_4^-$ concentrations in combination with low phenylephrine doses proved to have modulatory effects on transient frequency (results not shown). Maintaining a constant agonist concentration and increasing the $AlF_4^-$ concentration from 1 μM-$Al^{3+}$/1 mM-$F^-$ to 10 μM-$Al^{3+}$/5 mM-$F^-$ provoked a 27±6% increase in transient frequency without altering any other transient parameter.

In two cells adding $AlF_4^-$ (5 μM-$Al^{3+}$/5 mM-$F^-$ or less) to cells already exhibiting oscillations in response to phenylephrine proved to be inhibitory, as shown by prompt cessation of transients, and in one cell $AlF_4^-$ failed to evoke transients depsite the cell having already shown transients in response to phenylephrine (0.8 μM). Interestingly, not all perfusions yielded cells that were responsive to $AlF_4^-$ stimulation, despite the cells exhibiting transients to glycogenolytic stimuli; typically 50–60% of the cells were responsive to $AlF_4^-$. The physiological basis for this discrepancy is not known.

## DISCUSSION

Single rat hepatocytes respond to glycogenolytic agonists with series of repetitive free Ca oscillations [4, 12,13]. The characteristics of the oscillations are specific to agonist species and the transient frequency is closely related to hormone concentration [5]. This form of oscillatory behaviour arises as a direct result of the activation of the receptor–G-protein–PIC regulatory cycle. Stimulation of this pathway by receptor-independent means, using $AlF_4^-$, evokes free Ca oscillations similar to, but not identical with, those generated by agonists binding to receptors at the cell surface. The differences exist primarily in terms of periodicity, uniformity of transient waveform and in regularity of peak free Ca after $AlF_4^-$ stimulation. The active species producing oscillations in this study is most probably the fluoroaluminate ion, $AlF_4^-$, substituting for the γ-phos-

phate group of GTP in activating G-protein-liganded GDP. These results indicate that receptor occupancy is not a prerequisite for free Ca transient generation. However, the irregularity of the receptor-independent transients suggests that normally the agonist–receptor complex, or other associated factors, may have a modulatory role in transient generation.

Our observations of the prompt responses to $AlF_4^-$ are difficult to reconcile with an intracellular, G-protein-mediated, site of action. The concentration of $AlF_4^-$ required to activate G-proteins *in vivo* is comparable with that used here and it is conceivable that the transplasmalemmal influx of $AlF_4^-$ may be sufficiently fast to cause the prompt switch-on of spiking. However, we would expect a considerable delay upon $AlF_4^-$ wash-off, rather than the few minutes seen here, even after high $AlF_4^-$ concentrations. We therefore recognize that an extracellular site of action of $AlF_4^-$ may be more compatible with our observations of rapid wash-on and reasonably prompt wash-off of this agent, without being able to offer an explanation of its mechanism of action.

Previously we have suggested how repetitive free Ca transients may be generated in single rat hepatocytes [14]. The features of this model addressed here focus on the role of activated G-protein in stimulating PIC. Fluoroaluminate, at low concentrations, is known to activate PIC in rat hepatocytes [11]. The waveforms of the transients do not correspond consistently to that of any PI-dependent agonist species. In any one series of transients a broad spectrum of oscillation types is observed (see Fig. 2). Such an outcome would be expected if, as we believe ([14]; see also [15]), hepatocytes contain receptor-specific G-proteins and $AlF_4^-$ showed non-specificity in inducing G-protein activation. Indeed, we have argued that the differences in the transients, primarily in the falling phase, may occur as a result of the inactivation kinetics, which may be subtly different for each G-protein type [14].

Low concentrations of $AlF_4^-$, when added in combination with phenylephrine, prompt an increase in transient frequency without altering any of the other transient parameters. This outcome effectively mimics the result of increasing agonist concentration [4, 5], arguing strongly for the level of activated G-protein having a role in determining the intertransient interval.

The observation that $AlF_4^-$ induces oscillations and not a sustained increase in free Ca suggests that other elements of the oscillator feedback process continue to function in what may be an altered state of G-protein activation. Moreover, the finding that phorbol-ester-induced stimulation of protein kinase C inhibits $AlF_4^-$-induced free Ca changes in populations of hepatocytes [11] provides evidence for protein kinase-C-mediated G-protein (or PIC) phosphorylation having a role in negative feedback during the oscillator cycle [16]. It is clear that other factors must act to co-ordinate and regulate free Ca changes after stimulation; however, the identity of these crucial elements awaits further study.

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